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Award Number: DAMD17-01-1-0352

TITLE: Neural Protein Synuclein γ (SNCG) in Breast Cancer Progression

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REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Annual Summary (1 Jul 01 - 30 Jun 02)

4. TITLE AND SUBTITLE
Neural Protein Synuclein γ (SNCG) in Breast Cancer Progression

5. FUNDING NUMBERS
DAMD17-01-1-0352

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Synucleins are emerging as a central player in the fundamental neural processes and in the formation of pathologically insoluble deposits characteristic of Alzheimer's (AD) and Parkinson's (PD) diseases. Most studies of this group of proteins have been directed to the elucidation of their role in the formation of depositions in brain tissue. However, the normal cellular function of this highly conserved synuclein family remains largely unknown. In addition, synucleins have also been implicated in non-neural diseases particularly in the hormone-responsive cancers of breast and ovary. Using differential cDNA sequencing approach, we first identified a breast cancer specific gene, **BCSG1**, which was expressed abundantly in metastatic breast cancer cDNA library but scarcely in normal breast cDNA library. Interestingly, BCSG1 revealed no homology to any other known growth factors or oncogenes; rather, BCSG1 revealed extensive sequence homology to neurotic proteins of α synuclein and β synuclein, and thus was also named as γ Synuclein (SNCG). SNCG expression is highly associated with breast cancer and ovarian cancer progression. In addition, overexpression of SNCG in breast cancer cells significantly stimulated cell growth in vitro and tumor metastasis *in vivo*. However, the molecular targets of SNCG aberrant expression for breast cancer have not been identified. For the first time, we report a chaperone-like activity of SNCG in stimulating the transcriptional activity of estrogen receptor-α (ER-α) in MCF-7 cells. Consistent with the stimulation of ER-α, SNCG stimulated the ligand-dependent cell proliferation. Demonstration of the stimulation of ER-α signaling as one of the cellular functions of SNCG will have a great impact on the biology of steroid receptors and the pathological role of SNCG on hormone-responsive tumors including breast, ovary, and prostate.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer	8
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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A. INTRODUCTION

A-1. Identification of genes differentially expressed in breast cancer versus normal breast. We undertook a search, using the differential cDNA sequencing approach as we previously described (1-3), for isolation of differentially expressed genes in the cDNA libraries from normal breast and breast carcinoma. Of many putative differentially expressed genes, a breast cancer specific gene, BCSG1, which was **(a)** highly expressed in mammary gland relative to other organs and was **(b)** high abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library, was identified as a putative breast cancer marker (1). We demonstrated a stage-specific BCSG1 expression as follows: BCSG1 was undetectable in normal or benign breast lesions, showed partial expression in ductal carcinoma *in situ*, but was expressed at an extremely high level in advanced infiltrating breast cancer (1). Overexpression of BCSG1 was also demonstrated in ovarian cancer (4).

A-2. Neural protein synuclein. Interestingly, BCSG1 revealed no homology to any other known growth factors or oncogenes; rather, BCSG1 revealed extensive sequence homology to neurotic protein synuclein, having 54% and 56% sequence identity with α synuclein (SNCA) and β synuclein (SNCB), respectively. Subsequent to the isolation of BCSG1, synuclein γ (5) and persyn (6) were independently cloned from a brain genomic library and a brain cDNA library. In fact, BCSG1, SNCG, and persyn appear to be the same protein. Thus, the previously identified BCSG1, which is also highly expressed in brain (1), has been renamed as SNCG as the third member of synuclen family (7). Synucleins has been specifically implicated in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Mutations in SNCA is genetically linked to several independent familial cases of PD (8). More importantly, wild type of SNCA is the major component of Lewy bodies in sporadic PD (9-10). SNCA peptide known as non-amyloid component of plaques has been implicated in amyloidogenesis in AD (11-12). SNCB and SNCG have also been recognized to play a role in the pathogenesis of PD and Lewy bodies cases (13-14).

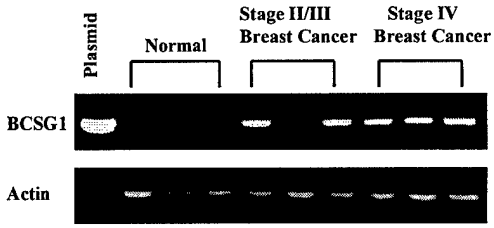
A-3. Expression of BCSG1/SNCG in breast and ovary cancer. Although synucleins are highly expressed in neuronal cells and are abundant in presynaptic terminals, synucleins have also been implicated in non-neural diseases particularly in the hormone responsive cancers of breast and ovary (1,4-5,15-17). Being identified as a breast cancer specific gene, SNCG mRNA was detected in neoplastic breast epithelial cells but not in normal mammary epithelial cells (1). While the expression of SNCG in normal breast is non-detectable (0 out of 7 normal breast specimens), 43% of stage II/III breast carcinomas (6 of 14) and 73% of stage IV breast carcinomas (11 of 15) expressed SNCG, respectively (**Fig. 1**). Western analysis to examine SNCG protein expression in human breast tissues showed a similar pattern in that it was not detected in normal breast tissues and stage I/II ductal breast carcinomas, but was detected in 70% of Stage III/IV ductal breast carcinomas (12). Ninkina et al were also able to confirm by using Northern and Western blotting that some breast tumors and breast tumor cell lines expressed SNCG, whereas normal breast tissue did not (16). In addition to the link to breast cancer progression, it has also been found that SNCG is involved in ovarian cancer. Following our identification of BCSG1, Lavedan et al first suggested that BCSG1/SNCG may be abnormally expressed in ovarian tumors as well as in breast tumors, based on the discovery of some SNCG ESTs in the libraries derived from an ovarian tumor (5). This suggestion was further confirmed by Western and immunohistochemical analyses (17). While synucleins (α, β, and γ) expression was not detectable by immunohistochemistry in normal ovarian epithelium, 87% (39 of 45) of ovarian carcinomas were found to express either SNCG or SNCB, and 42% (19 of 45) expressed all 3 synucleins (α, β, and γ) simultaneously.

B. RESEARCH REPORT

While the original aims of establishing the MMTV/SNCG transgenic mice is underway, we are a bit behind the schedul We are currently in the screening process. In addition, we have entered the new research direction and got some excitin new data.

Generation of SNCG transgenic mice. The full-length SNCG cDNA sequence from pCI-SNCG (4) was subcloned into the Bam HI and Apa I sites of pMMTV/STR (provided by Lynn Matrisian). A 3.5 kb XhoI MMTV-SNCG transgene was separated from the vector and isolated from an agarose gel. The DNA fragment was injected into fertilized eggs (5 ng/μl) at the Transgenic Core Facility at Albert Einstein College of Medicine. Transgenic mice will be generated according to standard procedures, and founder mice will be analyzed by PCR and Southern blot. Two lines will be selected for the following studies.

B-1. Specific expression of SNCG in advanced breast cancer. Our previous *in situ* hybridization analysis has demonstrated a stage-specific expression pattern of SNCG mRNA varying from virtually no detectable expression in normal or benign breast tissue to low level and partial expression in low grade *in situ* breast carcinoma to high expression in advanced infiltrating carcinomas (1). Because of the **non-quantitative** nature of the *in situ* analysis, we performed an RT-PCR analysis on 36 clinical breast specimens including normal or benign lesions, stage II/III breast carcinomas, and stage IV breast carcinomas. As shown in **Fig. 1**, while no SNCG mRNA was detectable in 7 breast specimens of normal or benign hyperplasia, SNCG mRNA was expressed in 43 % (6 of 14) and 73 % (11 of 15) of stage II/III and stage IV breast carcinomas, respectively.



Expression of BCSG1/SNCG in human breast

Stage	Normal (n=7)	Stage II/III (n=14)	Stage IV (n=15)
Expression	0 (0%)	6 (43%)	11 (73%)

Fig. 1. Expression of SNCG in human breast tissue. Total RNA was isolated from frozen human breast specimens. RT-PCR analysis of SNCG using primers within BCSG1 cording sequence (Sense: 5'-ATGGATGTCTTCAAGAAGGG-3'; antisense: 5'-CTAGTCTCCCCCACTCTGGG-3'). The 384-bp PCR product is a specific indication of the presence of SNCG. The integrity and the loading control of the RNA samples were ascertained by actin expression with a set of primers (5'-GCTGTGCTATCCCTGTACGC-3' and 5'-TGCCTCAGGGCAGCGGAACC-3') for 314-bp β-actin.

Using Western blot, Godwin AK's group also demonstrated a similar SNCG **protein** expression pattern in human breast samples. SNCG protein expression was not detectable in either normal breast or ductal carcinoma in situ (0 of 3) or Stage I/II breast carcinoma (0 of 6). However, 70% (12 of 17) of Stage III/IV breast carcinomas expressed SNCG protein. To emphasize the similarity and the importance of this stage-specific SNCG expression in breast tissue, PI downloaded Dr. Godwin's data here as Fig. 2 on SNCG expression in breast tumors.

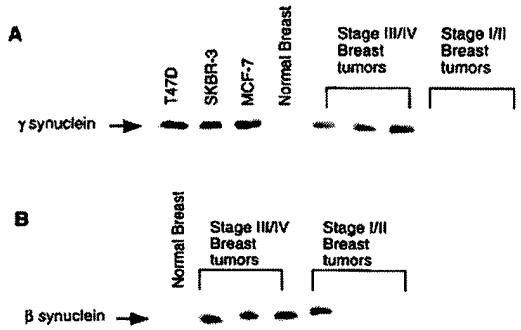


Fig. 2. Expression of SNCG and SNCB in breast (Cancer 88: 2154-2163, 2000). Tissue extracts were prepared and screened by Western blotting. (A) the blot was probed with the anti-SNCG antibody. (B) the blot was probed with the anti-SNCB antibody. Although SNCB was expressed in some breast carcinomas of all stages, including ductal carcinoma *in situ*, SNCG expression was restricted to advanced Stage III/IV breast carcinomas; 82% (14 of 17) of the Stage III/IV breast carcinomas expressed either SNCG, SNCB, or both simultaneously.

B-2. Overexpression of SNCG stimulated transcriptional activity of ER-α. To elucidate the molecular mechanisms underlying the abnormal transcription of SNCG in breast cancer cells, we previously isolated a 2195-bp promoter fragment of human SNCG gene and demonstrated that demethylation of exon 1 region of SNCG gene is an important factor responsible for the aberrant expression of SNCG in breast carcinomas (15). However, the molecular targets of SNCG aberrant expression for breast cancer have not been identified. Here we demonstrated ER-α as one of the critical target molecules for SNCG's action in breast cancer pathogenesis

We measured the effect of SNCG on modulating the transcriptional activity of ER-α in MC-7 human breast cancer cells. MCF-7 cells were transiently transfected with either the pCI-SNCG expressing plasmid or control pCI-neo plasmid. Transfection of SNCG gene into the SNCG-negative MCF-7 cells did not affect ER-α expression (**Fig. 3A**) but significantly stimulated E2-mediated activation of ER-α (**Fig. 3B**). While treatment of wild-type MCF-7 cells with 17-β-estradiol (E2) activated estrogen-responsive reporter ERE4-Luciferase (ERE4-Luc), overexpression of SNCG gene in MCF-7 cells increased E2-stimulated reporter activity 3.2-fold over the SNCG-negative control cells. The SNCG-stimulated transcriptional activity of ER-α was ligand-dependent, because SNCG had no significant effect on the transcriptional activity of ER-α in the absence of E2. Consistent with the increased transcriptional activity of ER-α, SNCG also stimulated E2-regulated gene transcription in MCF-7 cells (**Fig. 4**). While SNCG had no effect on

the transcription of Cathepsin D, PS2, and TGF-α in the absence of E2, transcription of Cathepsin D, PS2, and TGF-α were increased 4.6-fold, 3.3-fold, and 4.2-fold in SNCG transfected cells vs. control cells in the presence of E2, respectively.

B-3. Stimulation of cell proliferation by SNCG. To determine the biological relevance of SNCG-stimulated ligand-dependent ER-α signaling, we analyzed the effect of SNCG overexpression on the growth of breast cancer cells. The cellular proliferation of the previously established two stable SNCG-transfected MCF-7 cell clones, SNCG-MCF-2 and SNCG-MCF-6, were compared with that of SNCG-negative cells, neo-MCF-1 and neo-MCF-2 (11). Data in **Fig 5** shows that while SNCG had no effect on the proliferation of SNCG-MCF cells compared to neo-MCF cells in the absence of E2, overexpression of SNCG significantly stimulated the ligand-dependent proliferation. Treatment of neo clones with E2 stimulated average cell proliferation 2.4-fold over controls. However, E2 treatment of SNCG clones resulted in an average of 5.4-fold increase in the proliferation vs. controls, suggesting that SNCG expression renders the cells more responsive to E2-stimulated cell proliferation. Consistent with its stimulatory effect on ligand-dependent cell proliferation, overexpression of SNCG did not affect the proliferation of ER-α-negative MDA-MB-435 cells (9).

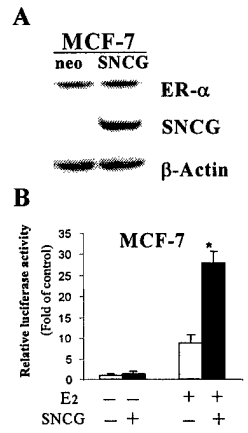


Fig. 3. SNCG stimulated ER-α transcriptional activity in MCF-7 human breast cancer cells. (**A**). Western analysis of ER-α and SNCG in MCF-7 cells transfected with pCI-SNCG or the control vector pCI-neo. Expression of SNCG did not affect the ER-α expression. (**B**). SNCG stimulated ER-α signaling MCF-7 cells. pERE4-Luc as well as control reporter pRL-SV40-Luc were co-transfected into SNCG-transfected and control neo-transfected cells. After transfection, cells were cultured in the ligand-free pheno-red free medium containing 5% stripped serum for 4 days, treated with or without 1 nM E2 for 24 hours before the promoter activities were determined by measuring the dual luciferase activity. The ERE reporter luciferase activity was normalized against the control renilla luciferase activity to correct for transfection efficiency. All values were presented as the fold induction over the control luciferase activity in the non-treated SNCG-negative cells, which was taken as 1. SNCG overexpression in ER-α-positive MCF-7 cells stimulated E2-activated reporter activity 3.2-fold over the SNCG-negative cells (*p<0.001).

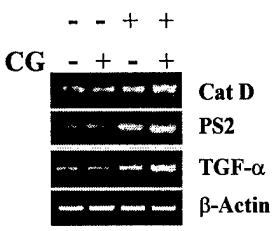


Fig. 4. SNCG stimulated estrogen-regulated gene transcription in MCF-7 cells. Cells were cultured in the ligand-free medium for 4 days. Cells were then treated with or without 1 nM of E2 for 8 hours before the isolation of mRNA. Expressions of mRNA of Cathepsin D (Cat-D), PS2, and TGF-α were studied in SNCG transiently transfected cells vs. control cells by RT-PCR analyses. A 842-bp product of Cat-D, a 336-bp product of PS2, and a 240-bp product of TGF-α, were amplified by RT-PCR.

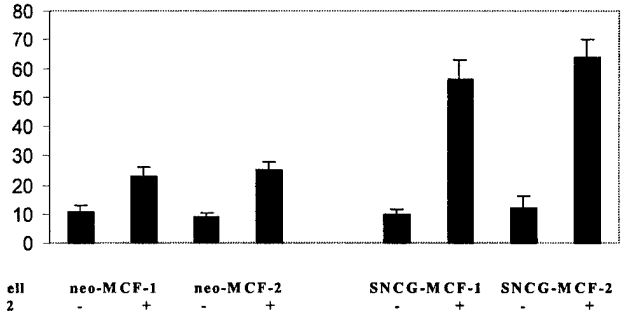


Fig. 5. SNCG stimulated ligand-dependent cell proliferation. Cells were cultured in the ligand-free Conditioned Cell Culture for 4 days and then treated with or without 10 nM E2 for 24 hours. Cell proliferation was measured by ^{3}H- thymidine incorporation. Data are means ± SD of three cultures.

C. KEY RESEARCH ACCOMPLISHMENTS

1. In transient transfection assays in human breast cancer cells, SNCG strongly stimulated the ligand-dependent transcriptional activity of ER-α. The SNCG-stimulated ER-α signaling was demonstrated in SNCG-transfected ER-α-positive and SNCG-negative MCF-7 cells.

2. Consistent with the chaperone activity in stimulation of ER-α, SNCG stimulated the ligand-dependent cell proliferation.

D. CONCLUSIONS

1. Although synucleins are highly expressed in neuronal cells and are abundant in presynaptic terminals, synucleins have also been implicated in non-neural diseases particularly in the hormone-responsive cancers of breast and ovary. SNCG was first identified by differential cDNA sequencing as a breast cancer specific gene, which was expressed abundantly in metastatic breast cancer cDNA library but scarcely in normal breast cDNA library. SNCG expression is highly associated breast cancer and ovarian cancer progression. In addition, overexpression of SNCG in breast cancer cells significantly stimulated cell growth in vitro and tumor metastasis *in vivo*. However, the molecular targets of SNCG aberrant expression for breast cancer have not been identified. Here we demonstrated ER-α as one of the critical target molecules for SNCG's action in breast cancer pathogenesis. Our findings suggest that SNCG may function, at least in part, by participating in Hsp90-based multiprotein chaperone system for efficient activation of steroid receptors. Thus, aberrant expression of SNCG stimulates breast cancer growth and progression by enhancing the transcriptional activity of ER-α.
2. Synucleins are emerging as a central player in the fundamental neural processes and in the formation of pathologically insoluble deposits characteristic of Alzheimer's (AD) and Parkinson's (PD) diseases. Most studies of this group of proteins have been directed to the elucidation of their role in the formation of depositions in brain tissue. However, the normal cellular function of this highly conserved synuclein family remains largely unknown. Here we demonstrated that one of the functions of SNCG is activating ER-α signaling. The preventive effect of estrogen on AD has become clear with epidemiological data, suggesting that estrogen may act as a neuroprotectant against the neurodegenerative diseases. The demonstration of ER-α as one of the critical target for SNCG-mediated chaperone activity may indicate a new direction of normal cellular function of synucleins. In this regard, SNCG may be involved in mediating the function of transcriptional activity of ER-α in neuronal cells, thus, the loss or decreased SNCG expression may lower the beneficial effects of estrogen to protect neurons against PD and AD. The potential role of SNCG as a neuroprotectant warrants further investigation.

E. REFERENCES

1. Ji H, Liu YE, Jia T, Wang M, Liu J, Xiao G, Joseph BK and Shi YE. Identification of a breast cancer-specific gene, BCSG1, by direct differential complementary DNA sequencing. ***Cancer Res.***, 57: 759-764, 1997.
2. Shi YE, Ni J, Xiao G, Liu YE, Fuchs A, Yu G, Zhang M, Li J, Aggarwal BB, Meager A and Gentz R. Antitumor activity of the novel human breast cancer growth inhibitor MRG. ***Cancer Res.***, 57 (15): 3084-3091, 1997.
3. Xiao G, Liu YE, Ni J, Gentz R, Sang QA and Shi YE. Suppression of breast cancer growth and metastasis by a novel serpin MEPI that is expressed specifically in the mammary myoepithelial cells. ***PNAS*** 96 (7): 3700-3705, 1999.
4. Jia T, Liu J, Liu YE and Shi YE. Stimulation of breast cancer invasion and metastasis by synuclein γ (SNCG). ***Cancer Res.*** 59: 742-747, 1999.
5. Lavedan, C., Leroy, E., Dehejia, A., Buchholtz, S., Dutra, A., Nussbaum, R. L., and Polymeropoulos, M. H. (1998). Identification, localization and characterization of the human gamma- synuclein gene. Hum Genet *103*, 106-112.
6. Ninkina, N. N., Alimova-Kost, M. V., Paterson, J. W., Delaney, L., Cohen, B. B., Imreh, S., Gnuchev, N. V., Davies, A. M., and Buchman, V. L. (1998). Organization, expression and polymorphism of the human persyn gene. Hum Mol Genet *7*, 1417-1424.
7. Clayton, D. F., and George, J. M. (1998). The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. Trends Neurosci *21*, 249-254.
8. Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., *et al.* (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science *276*, 2045-2047.
9. Spillantini, M. G., Schmidt, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997). Alpha-synuclein in Lewy bodies. Nature *388*, 839-840.
10. Takeda, A., Mallory, M., Sundsmo, M., Honer, W., Hansen, L., and Masliah, E. (1998). Abnormal accumulation of NACP/alpha-synuclein in neurodegenerative disorders. Am J Pathol *152*, 367-372.

11. Ueda, K., Fukushima, H., Masliah, E., Xia, Y., Iwai, A., Yoshimoto, M., Otero, D. A., Kondo, J., Ihara, Y., and Saitoh, T. (1993). Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease. Proc Natl Acad Sci U S A *90*, 11282-11286.
12. Yoshimoto, M., Iwai, A., Kang, D., Otero, D. A., Xia, Y., and Saitoh, T. (1995). NACP, the precursor protein of the non-amyloid beta/A4 protein (A beta) component of Alzheimer disease amyloid, binds A beta and stimulates A beta aggregation. Proc Natl Acad Sci U S A *92*, 9141-9145.
13. Duda, J. E., Lee, V. M., and Trojanowski, J. Q. (2000). Neuropathology of synuclein aggregates. J Neurosci Res *61*, 121-127.
14. Galvin, J. E., Uryu, K., Lee, V. M., and Trojanowski, J. Q. (1999). Axon pathology in Parkinson's disease and Lewy body dementia hippocampus contains alpha-, beta-, and gamma-synuclein. Proc Natl Acad Sci U S A *96*, 13450-13455.
15. Liu A, Gupta A, Li C, Ahlborn TE, Ma Y, **Shi YE** and Liu J. Molecular Mechanisms for Aberrant Expression of the Human Breast Cancer Specific Gene 1 in Breast Cancer Cells: Control of transcription by DNA methylation and intronic sequences. Oncogene 20 (37): 5173-5185, 2001.
16. Liu J, Spence MJ, Zhang YL, Jiang Y, Liu YE and **Shi YE**. Transcriptional suppression of synuclein γ (SNCG) expression in human breast cancer cells by the growth inhibitory cytokine oncostatin M. Breast Cancer Research and Treatment 62:.99-107, 2000.
17. Bruening, W., Giasson, B. I., Klein-Szanto, A. J., Lee, V. M., Trojanowski, J. Q., and Godwin, A. K. (2000). Synucleins are expressed in the majority of breast and ovarian carcinomas and in preneoplastic lesions of the ovary. Cancer *88*, 2154-2163.

F. REPORTABLE OUTCOME

Abstract: Yangfu Jiang and Yuenian E. Shi. (2002). BCSG1/gamma synuclein: a new marker for breast cancer progression. Proceedings of the American Association for Cancer Research. 43: 713.